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Thermal Inactivation of Infectious Hepatitis E Virus in Experimentally Contaminated Food

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Hepatitis E virus (HEV) infection of zoonotic origin is an emerging concern in industrialized countries. In the past few years, several cases of zoonotic hepatitis E have been identified and the consumption of food products derived from pork liver have been associated with clusters of human cases. More specifically, raw or undercooked pork products have been incriminated. Few data on the effect of heating on HEV inactivation in food products are available. In the present study, the various times and temperatures that are used during industrial processing of pork products were applied to experimentally contaminated food preparations. After treatment, the presence of residual infectious virus particles was investigated using real-time reverse transcription-PCR and an *in vivo* experimental model in pigs. Results show that heating the food to an internal temperature of 71°C for 20 min is necessary to completely inactivate HEV. These results are very important for determining processing methods to ensure food safety in regard to food-borne hepatitis E.

Hepatitis E virus (HEV) infections are responsible for large epidemics of acute viral hepatitis in several developing countries in tropical and subtropical regions. In addition, sporadic cases of hepatitis E have also been reported in the United States, Japan, and Europe. HEV is becoming the first cause of enterically transmitted hepatitis in humans.

The disease caused by HEV is typically characterized as self-limiting acute hepatitis with low mortality. However, severe hepatitis has been reported in pregnant women, with up to 20% mortality (23). A significant proportion of healthy individuals in industrialized countries are seropositive for HEV, and a high prevalence of anti-HEV antibodies of more than 20% has been reported in some areas of the United States (18). Anti-HEV antibodies have also been detected in many animal species, and HEV RNA has been isolated from domestic pigs and wild animals (boars, deer, and mongoose). HEV is the only hepatitis virus that infects animals other than primates (22).

The virus is a nonenveloped, single-stranded, positive-sense RNA virus, classified in the *Hepevirus* genus of the *Hepeviridae* family (11). HEV sequences isolated worldwide can be classified into four major genotypes. Genotypes 1 and 2 have been reported in humans from Asia and Africa and from Mexico. Genotypes 3 and 4 have been identified in both humans and swine in industrialized countries as well as in Asia (23).

In regions of endemicity, the main transmission pathway of hepatitis E virus is through consumption of contaminated water or spoiled food. In contrast, in areas of nonendemicity, ingestion of raw or undercooked contaminated deer and boar meat has been associated with sporadic cases of acute hepatitis E in humans (19, 26). Furthermore, in several countries, 2 to 11% of pork livers on the market or at slaughterhouses are contaminated by HEV, and some contain infectious virus particles (2, 13, 25, 27). More recently, in France, several cases of hepatitis E were associated with the consumption of sausages made from raw pork liver (4), and HEV genotype 3 was detected in 7 out of 12 sausage samples. Thus, hepatitis E is considered a food-borne disease. The zoonotic potential of HEV has also been confirmed using animal models. HEV genotype 3 isolated from swine can cross the species barrier and infect primates after experimental inoculation (21). Accordingly, pigs can be effectively experimentally infected with human HEV genotype 3 or 4 (20, 22).

Since HEV is associated with consumption of raw pork products, it is important to determine if heating would be an efficient method for inactivating HEV and reducing the risk of HEV exposure. Few data on HEV resistance to thermal treatment are available. The two available studies on HEV thermal inactivation used different *in vitro* or *in vivo* models. The first study was based on heating of fecal suspensions of HEV genotypes 1 and 2 to temperatures between 45 and 70°C and inoculation in a cell culture permissive to HEV (12). The second study used pigs inoculated with pork liver homogenates containing infectious HEV of genotype 3 heated to 56°C by frying or boiling (14). Both studies show that HEV is more likely to resist heating to 56°C and is inactivated at temperatures greater than 71°C. These results raise questions on what the fate of HEV would be during industrial processing using temperatures within this range (i.e., 56°C to 71°C). Moreover, these studies did not address the thermal resistance of HEV in food products made up of complex meat matrices and fat.

Thus, to estimate the time and temperature required to inactivate HEV in pork products, contaminated products were fabricated from HEV-infected liver and underwent different processing methods used by the food industry. The quantity of HEV was estimated using quantitative reverse transcription-PCR (qRT-PCR). The presence of residual infectious virus particles in food products after heat treatments was assessed using pigs as an *in vivo* model of experimental infection.

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MATERIALS AND METHODS

Virus and HEV-infected liver samples. Pig liver containing HEV genotype 3, subtype 3e (GenBank accession number EF494700), was collected from an experimentally infected pig. The level of HEV contamination of the liver was estimated to be 10^8 copies of HEV genome equivalents (GE)/g using real-time qRT-PCR as described below. Liver samples of 100 g were stored with no additives or preservatives at −80°C until further processing.

Food sample processing. Infected livers (30%) were homogenized with fat (48%) and warm water (17%) using a food processor (Robocoupe, Montceau-en-Bourgogne, France) to obtain an emulsion. Then, spices (0.5%), nitrite salt (2%, containing 99.4% sodium chloride and 0.6% sodium nitrite E250), dextrose (0.5%), and sodium caseinate (2%) were added and the mixture was further homogenized to obtain a pâté-like preparation. For the negative control, the same preparation was made using noninfected pork liver collected from a noninfected animal used in the same experimental infection mentioned above. Then, equal portions (25 g) of the pâté-like preparation, 10 infected samples and 1 noninfected sample, were sealed in a sterile plastic bag and flattened to a thickness of 2 mm.

Thermal treatment of food samples. Each sealed food preparation was incubated in a controlled water bath using three different temperatures and three durations per temperature. Three temperatures commonly used in industrial food possessing were selected: 62, 68, and 71°C. For each temperature, three incubation times were applied: 5, 10, and 20 min for the 68°C and 71°C water baths or 5, 20, and 120 min for the 62°C water bath. Temperatures were monitored using a data logger thermometer (HH147U; Omega, Stanford, CA) (Fig. 1) placed in a control sample made of noninfected liver. After heating, the samples were immediately cooled using a cold water bath containing ice.

Preparation of inocula for experimental infection. After the different heating treatments, the samples were homogenized in phosphate-buffered saline (PBS; pH 7.2) to prepare viral suspensions as follows. Five grams of the pâté-like preparation was homogenized on ice with 15 ml of cold PBS, using an Ultra-Thurax T18 homogenizer (IKA, Mac Technologie, Gretz Armainvilliers, France). After centrifugation at 1,500 × *g* for 15 min at 4°C, the supernatant was collected, aliquoted, and stored at −20°C until inoculation. HEV genome quantification was performed using qRT-PCR as described below.

Swine bioassay. Forty specific-pathogen-free (SPF) pigs (free from HEV) were divided into 11 groups of 3, 4, or 6 pigs. Four rooms (level 3 biosafety facilities with air filtration) were used. For rooms with two pens, full partitions were put between the pens to prevent contact and spread of fecal material from one pen to another. For space and practical reasons, some groups of animals were handled in the same pens. Groups for which heating time was the longest for a given temperature were made up of 4 pigs housed in a single pen. Negative and positive controls were housed separately from the other groups (in independent rooms). All animals were inoculated intravenously in the ear with 2 ml of viral suspensions. Groups 1 (3 pigs), 2 (3 pigs), and 3 (4 pigs) were inoculated with a suspension prepared from the pâté-like preparation heated to 71°C for 5, 10, and 20 min, respectively. Pigs in groups 4 (3 pigs), 5 (3 pigs), and 6 (4 pigs) were inoculated with the suspension of the pâté-like preparation heated to 68°C for 5, 10, and 20 min, respectively. Pigs in groups 7 (3 pigs), 8 (3 pigs), and 9 (4 pigs) were inoculated with the suspension of the pâté-like preparation heated to 62°C for 5, 20, and 120 min, respectively. Group 10 (4 pigs) was inoculated with an unheated suspension of the pâté-like preparation as a positive control of infection. Group 11 (6 pigs) was not inoculated with the virus suspension and thus served as a negative control. To follow the three R's (reduction, refinement, and replacement) of animal experimentation, these negative controls were shared with another experimental procedure and did not receive any injection. Animals were monitored for 5 weeks, except for the animal controls, which were monitored for only 2 weeks. Evidence of HEV infection was followed by detecting viral excretion in fecal samples and anti-HEV antibodies in sera. Fecal samples were collected three times a week, and serum samples were collected once a week. After 35 days, all animal were necropsied. This experimental protocol was validated by the Ethical Committee ComEth (reference number 10-0026) of the National Veterinary School of Alfort, the National Agency for Food Safety, and the University of Paris 11. This protocol was performed according to the safety procedures required for hepatitis E. Pig handlers wore safety gloves, goggles, specific clothes, waterproof trousers, boots, and FFP2 masks to ensure proper individual protection. Cross-contamination between pens was prevented by thorough washing of boots and waterproof trousers before entering each pen and by using material specific to each pen.

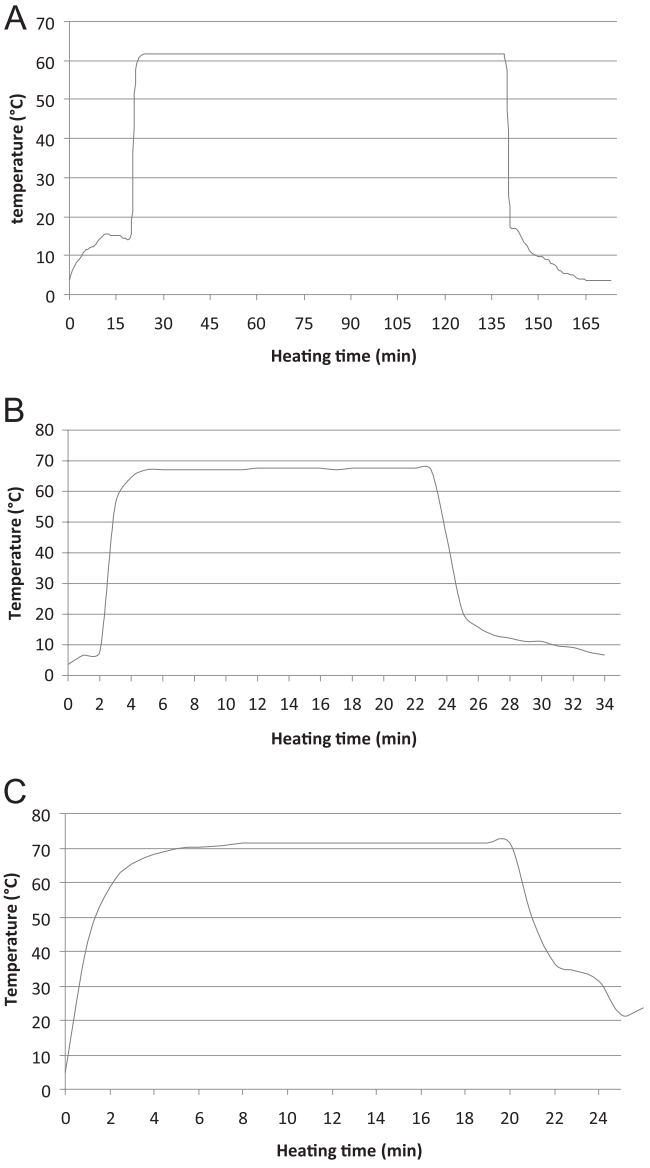

FIG 1 Monitoring of internal temperature of the pâté-like preparation. For each temperature, a thermal sensor was used to measure the internal temperature of samples consisting of a control pâté-like preparation made with noninfected pork liver. For a given temperature, all samples were immersed simultaneously and then removed sequentially at the chosen duration of incubation. (A) 62°C; (B) 68°C; (C) 71°C.

Nucleic acid isolation. Viral RNA was extracted using the commercially available QIAamp viral RNA minikit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol, except that extraction was per-

formed on 200 μl of serum, viral suspension (33% PBS, pH 7.2), or fecal sample (10% PBS, pH 7.2) with 800 μl of AVL buffer with carrier. RNA was eluted twice with 40 μl of sterile water after 1 min of incubation at room temperature. RNA was aliquoted and stored at −80°C until use.

Controls. Precautions were taken to prevent false-positive and false-negative results. In addition to spatial separation of work spaces at crucial experimental points (e.g., RNA extraction and PCR mix preparation), each experiment included several overall control samples.

HEV RNA detection. HEV RNA detection was performed through genomic amplification using a qualitative method based on nested RT-PCR or a quantitative method using a TaqMan real-time RT-PCR assay.

(i) RT-nested PCR. To detect HEV RNA in fecal and serum samples from inoculated pigs, a nested RT-PCR assay was adapted from a method previously described by Cooper et al. (5). Nested RT-PCR amplifies a region within the ORF2 capsid gene from nucleotides 5996 to 6343. Briefly, reverse transcription was performed at 42°C for 60 min with 5 μl of total RNA (template), 2.5 μl of random hexamer, 60 U of PrimeSript Reverse Trans TaKaRa in RT buffer (Ozyme, St Quentin-en-Yvelines, France), 1 mM deoxynucleoside triphosphate mix (Ozyme, St.-Quentin-en-Yvelines, France), and 12 U of RNase inhibitor (Life Technologies, Villebon-sur-Yvette, France). The first round of PCR was performed with a set of degenerate HEV primers: 3156N [forward, 5′-AATTATGCC(T)C AGTAC(T)CGG(A)GTTG-3′] and 3157N [reverse, 5′-CCCTTA(G)TCC (T)TGCTGA(C)GCATTCTC-3′]. Reactions were performed in a 25-μl reaction mixture containing 2 μl of the resulting cDNA (template), 1 U of Platinum DNA polymerase (Life Technologies, Villebon-sur-Yvette, France) in 10× Platinum buffer, 1.5 μl of $MgCl_2$ mix (50 mM), and 0.2 μM each 3156N forward primer and 3157N reverse primer. The settings of this first PCR were denaturation at 94°C for 1 min, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 52°C, and an extension at 72°C for 30 s and then by 10 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 40°C, and a final extension at 72°C for 30 s, followed at the end by a final extension at 72°C for 10 min.

The second round of PCR was performed with another set of degenerate HEV primers: 3158N [forward, 5′-GTT(A)ATGCTT(C)TGCATA (T)CATGGCT-3′] and 3159N (reverse, 5′-AGCCGACGAAATCAATTCT GTC-3′). All oligonucleotides were purchased from MWG Biotech AG (Eurofins MWG, Ebersberg, Germany). Reactions were performed in a 50-μl final reaction mixture containing 5 μl of the resulting first-round PCR product (template), 1 U of Platinum DNA polymerase (Life Technologies, Villebon-sur-Yvette, France) in 10× Platinum buffer, 1.5 μl of $MgCl_2$ mix (50 mM), and 0.2 μM each 3158N forward primer and 3159N reverse primer. The PCR settings for the second round were an initial denaturation step at 94°C for 1 min, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 52°C, and an extension at 72°C for 30 s and then by a final extension at 72°C for 10 min. The expected final product of the nested RT-PCR was 348 bp. Amplified products were visualized after migration on an agarose (0.8%) gel and ethidium bromide staining (24). Negative controls of RT, the first PCR, and nested PCR were included in each run.

(ii) TaqMan real-time RT-PCR assay. HEV RNA quantification was adapted from a previously described method (16). Briefly, TaqMan RT-PCR was performed using a QuantiTec Probe RT-PCR kit (Qiagen) according to the manufacturer's instructions with 2 μl of RNA (template). Reverse primer (5′-AGGGGTTGGTTGGATGAA-3′) was used at a final concentration of 0.25 μM, forward primer (5′-GGTGGTTTCTGGGGT GAC-3′) was used at a final concentration of 0.1 μM, and probe (FAM-TGATTCTCAGCCCTTCGC-MGB, where FAM is 6-carboxyfluorescein and MGB is major groove binder) was used at a final concentration of 5 μM. A LightCycler apparatus (Roche Molecular Biochemicals, Meylan, France) was used for sample analysis. RT was carried out at 50°C for 20 min, followed by denaturation at 95°C for 15 min. DNA was immediately amplified with 45 cycles at 95°C for 10 s and 58°C for 45 s. The final extension was followed by cooling at 40°C for 30 s. Real-time RT-PCR data were collected after the reaction and the crossing points (CPs) were calculated. For generation of standard quantification curves, the CP values were plotted against the logarithm of the input copy numbers of standard RNA. Standard RNA was obtained after *in vitro* transcription of plasmid pCDNA 3.1 ORF2-3 HEV, using a MegaScript kit Ambion (Life Technologies, Villebon-sur-Yvette, France) according to the manufacturer's instructions. RNA was then purified using an RNeasy kit according to the manufacturer's instructions (Qiagen). RNAs were quantified using a Nanodrop apparatus (Life Technologies, Villebon-sur-Yvette, France). The standard plasmid was constructed by cloning into NheI/XhoI-digested pCDNA 3.1 (Life Technologies, Villebon-sur-Yvette, France) a fragment corresponding to the genomic region from nucleotides 5190 to 5489 of the French swine HEV genotype 3f sequence (GenBank accession number JF718793). Amplification and cloning were performed using forward primer 5′-NheI-CTGCATCGCCCATGGGATCGC-3′ and reverse primer 5′-XhoI-CGCTGGGACTGGTCACGCC-3′. The presence of RT-PCR inhibitors was estimated by overloading the samples with 10^5 copies of standard HEV ARN separately. Each overloaded sample was analyzed in duplicate. The recovery rate was calculated as the ratio of GE quantity observed/quantity of GE spiked (10^5). Then, for each sample analyzed, the quantity of HEV GE was corrected with the recovery rate of each overload (Table 1).

TABLE 1 HEV quantification after heat treatments

Group	Heat treatment		Overload recovery rate[a]	HEV GE g^{-1}	Log reduction[b]	GE inoculated
	Temp (°C)	Time (min)				
1	71	5	0.41	2.60×10^4	2.93	6.50×10^4
2	71	10	0.52	5.90×10^4	2.58	1.15×10^5
3	71	20	0.45	4.60×10^4	2.69	1.01×10^5
4	68	5	0.44	1.17×10^5	2.28	2.34×10^5
5	68	10	0.37	1.23×10^5	2.26	2.46×10^5
6	68	20	0.30	1.10×10^5	2.31	2.49×10^5
7	62	5	0.40	1.43×10^6	1.19	2.86×10^6
8	62	20	0.38	3.33×10^5	1.83	6.93×10^5
9	62	120	0.31	1.52×10^5	2.17	3.04×10^5
10	None	None	0.97	2.24×10^7		3.44×10^7

[a] Overload recovery rate corresponds to the GE quantification of the overload obtained/GE overload expected.

[b] Log reduction corresponds to the initial titer (in log units) of unheated sample (group 10) − GE load (in log units) for each sample.

HEV serology. A commercial test validated for veterinary analysis was used to detect anti-HEV antibodies in pigs: HEV enzyme-linked immunosorbent assay (ELISA; version 4.0; MP Diagnostics, Illkirch, France). This test is based on a double-sandwich ELISA that allows the detection of all classes of immunoglobulin (IgG and IgM) regardless of animal species. The HEV ELISA (version 4.0) utilizes a proprietary recombinant antigen which is highly conserved between different HEV strains. Analyses were performed according to the manufacturer's instructions, except that 10 μl of serum was used. Samples were considered positive when the optical density (OD) at 450 nm of the sample was superior to the cutoff value (cutoff value = mean of the negative control + 0.300).

Statistical analysis. The impact of heating time and temperature on the viral genome load reduction (log scale) was first assessed. The viral genome load reduction was calculated as the difference between the genome load equivalent of the nontreated preparation and that of the heated preparation. Linear regression was used to relate the genome load reduction to temperature, time, and the interaction. Heating time was also converted to the log scale in this analysis.

In a second step, the effects of heating time and temperature on three factors were assessed: (i) the frequency of HEV-positive pigs, (ii) the duration of HEV shedding, and (iii) the time to HEV infection. Logistic regression was used to relate the probability of infection to the effect of

TABLE 2 HEV fecal excretion in experimentally infected pigs

Group	Temp (°C)	Time	No. of pigs excreting HEV/no. of pigs in group on the following day postinoculation:														
			−4	2	4	7	9	11	14	16	18	22	25	28	30	32	35	Total
1	71	5	0/3	0/3	0/3	0/3	0/3	2/3	2/3	2/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	2/3
2	71	10	0/3	0/3	0/3	0/3	0/3	0/3	0/2[a]	0/3	1/3	2/3	2/3	1/3	1/3	1/3	0/3	2/3
3	71	20	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/3[a]	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
4	68	5	0/3	0/3	0/3	0/3	1/3	1/3	1/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/2[a]	1/3
5	68	10	0/3	0/3	0/3	0/3	1/3	1/3	0/3	2/3	1/3	1/3	1/3	0/3	0/3	0/3	0/3	2/3
6	68	20	0/4	0/4	0/4	0/4	1/4	1/4	3/4	3/4	2/4	1/4	0/4	0/4	0/4	0/4	0/4	3/4
7	62	5	0/3	0/3	0/3	0/3	3/3	3/3	2/3	2/3	1/3	1/3	1/3	1/3	1/3	1/3	1/3	3/3
8	62	20	0/3	0/3	0/3	1/3	3/3	3/3	3/3	3/3	1/2[a]	2/3	2/3	1/3	0/3	0/3	0/3	3/3
9	62	120	0/4	0/4	0/4	0/4	1/4	1/4	2/4	3/4	3/4	3/4	1/4	1/4	0/4	0/4	0/4	3/4
10	HEV positive, no heating		0/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	3/4	3/4	3/4	3/4	3/4	4/4
11			0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/3	—[b]	—	—	—	—	—	—	0/6

[a] One sample was missing.
[b] —, no samples were taken.

temperature, heating time, and their interaction. Due to the small sample size, the nonparametric Kruskal-Wallis test was used to assess the effect of heating time for each temperature on the duration of excretion. The times to HEV excretion and seroconversion were studied using survival analysis, with the time at first excretion or the first detection of anti-HEV antibodies being the time-to-event variable. A semiproportional hazard Cox model was used to relate time to first excretion and time to seroconversion with the independent variables of temperature, heating time, and their interaction. Statistical analyses were performed using SAS (version 9.1) statistical analysis software (SAS/STAT User's Guide, 2002; SAS Institute Inc., Cary, NC).

RESULTS

Quantification of HEV after heat treatments. The numbers of GE of HEV detected in food products prepared experimentally with HEV-contaminated liver and heated to different temperatures for different durations are shown in Table 1. Estimation of RT-PCR inhibitors by overloading samples with a known quantity of HEV RNA showed recovery rates of between 0.30 and 0.52 in heated samples and 0.97 for the nonheated sample (Table 1) or liver (data not shown). The initial contamination of the infected pâté-like preparation was estimated to be 2.24×10^7 HEV GE g^{-1}. After treatment at 71°C for 5, 10, or 20 min, the HEV titer decreased to 2.60×10^4, 5.90×10^4, and 4.60×10^4 GE g^{-1}, respectively. Heating to 68°C also reduced the HEV titers for all three durations (5, 10, or 20 min) to 1.17×10^5, 1.23×10^5, and 1.10×10^5 GE g^{-1}, respectively. Finally, heating to 62°C for 5, 20, and 120 min reduced the HEV titer to 1.43×10^6, 3.33×10^5, and 1.52×10^5 GE g^{-1}, respectively. Heating had an effect on the apparent HEV titer in terms of GE, with a log reduction of nearly 3 log units. From the linear model, both heating time and temperature had a significant positive effect on viral genome load reduction ($P < 0.001$). There was also a significant negative interaction between heating time and temperature ($P < 0.001$).

HEV excretion in experimentally infected pigs. The presence of infectious HEV particles in the inocula was evaluated by detecting fecal HEV excretion in inoculated animals (Table 2). Fecal samples were collected from 1 to 35 days postinoculation (dpi). None of the six negative-control pigs excreted HEV from −4 to day 16 dpi. All four positive-control pigs started to excrete HEV at 2 dpi until 22 dpi or the end of the experiment (35 dpi). For pigs inoculated with the suspensions from the treatments consisting of 5, 20, and 120 min at 62°C, all pigs in each group, except one at 120 min, were infected, with the onset of HEV excretion occurring at 7 to 9 dpi and lasting until 22 to 35 dpi. For the 68°C treatment, one to two pigs per group were infected, excreting HEV from 9 to 16 dpi until 16 to 22 dpi. Of the 10 pigs inoculated with the viral suspension heated to 71°C, the two groups of three animals inoculated with the 5- or 10-min treatments were infected. Viral excretion started at 11 dpi and lasted until day 16 or 18 dpi in the 5 min/71°C treatment group. In the 10 min/71°C group, viral excretion started at 18 or 22 dpi and lasted until 25 or 32 dpi. In the group of four pigs inoculated with the viral suspension from the pâté-like preparation heated to 71°C for 20 min, none of the pigs excreted HEV, suggesting that HEV was inactivated. To confirm that these four pigs were not at all infected, HEV RNA detection was performed on sera and liver on the day of necropsy (35 dpi). All samples were HEV negative (data not shown).

The effect of temperature was significantly related to the probability of infection (logistic regression model, $P = 0.04$), with the odds of HEV infection being significantly reduced at 71°C compared to that at 62°C. Neither the duration of the heat treatment nor the interaction between temperature and heating time was significant ($P > 0.05$).

From the nonparametric Kruskal-Wallis test, no significant relationship between duration of excretion and heating temperature or heating time was found.

Conversely, the time to first excretion was significantly delayed for the highest temperature (71°C) compared to that for 62°C ($P = 0.02$). Heating time was not significantly related to time to excretion in the survival analysis.

HEV seroconversion in experimentally infected pigs. The detection of anti-HEV antibodies was performed on all groups of animals until 35 dpi. None of the negative controls showed anti-HEV antibodies before 14 dpi (Table 3). In contrast, three of four pigs infected in the HEV-positive-control group had an anti-HEV response. The ELISA used for detection of anti-HEV antibodies is based on a double-sandwich technique which detects all classes of immunoglobulins, including IgM and IgG. Thus, for one pig in this group (pig 2870), the first peak was detected at 14 dpi and probably corresponded to IgM; the second peak occurred at 35 dpi and corresponded to IgG. For the two other animals, one major peak was observed at 35 dpi. One infected animal (pig 2886) of this

TABLE 3 HEV seroconversion in experimentally infected pigs

Group	Temp	Time	No. of pigs seroconverting/no. of pigs in group on the following day postinoculation:						
			−4	4	7	14	22	28	35
1	71	5	0/3	0/3	0/3	0/3	2/3	2/3	1/3
2	71	10	0/3	0/3	0/3	0/3	0/3	1/3	2/3
3	71	20	0/4	0/4	0/4	0/4	0/4	0/4	0/4
4	68	5	0/3	0/3	0/3	0/3	1/3	0/3	1/3
5	68	10	0/3	0/3	0/3	0/3	2/3	1/3	0/3
6	68	20	0/4	0/4	0/4	0/4	3/4	3/4	2/4
7	62	5	0/3	0/3	0/3	1/3	1/3	1/3	1/3
8	62	20	0/3	0/3	0/3	0/3	2/3	3/3	1/3
9	62	120	0/4	0/4	0/4	0/4	1/4	3/4	2/4
10	HEV positive, no heating		0/4	0/4	0/4	2/4	1/4	3/4	3/4
11			0/6	0/6	0/6	0/6	—[a]	—	—

[a] —, no samples were taken.

group did not show any anti-HEV antibodies at 35 dpi (Table 3; Fig. 2). In the group inoculated with samples heated to 62°C, only one of the three infected pigs in the 5-min treatment group became positive for anti-HEV antibodies at 14 dpi. In the treatment groups consisting of 20 min and 120 min at 62°C, all infected animals (3/3 and 4/4) had anti-HEV antibodies at 28 dpi. For some animals, the titer of anti-HEV antibodies, evaluated by OD, decreased to under the cutoff value at 35 dpi, which may correspond to the IgM-IgG class switch observed in the positive-control group (Table 3; Fig. 2). In the group inoculated with samples heated to 68°C, all infected pigs (1/3, 2/3, and 3/4 in the 5-min, 10-min, and 20-min, groups, respectively) had anti-HEV antibodies at 22 dpi. Later on, the titer of anti-HEV antibodies evaluated using OD values decreased for most samples (Table 3; Fig. 2). In the group inoculated with samples heated to 71°C, all infected pigs (2/3) in the 5- and 10-min treatments had anti-HEV antibodies at 35 dpi. The two pigs in the 5-min/71°C group showed anti-HEV antibodies at 22 dpi, and the two pigs in the 10-min/71°C group showed anti-HEV antibodies only at 28 or 35 dpi (Table 3; Fig. 2). The effect of temperature was significantly related to the probability of seroconversion (logistic regression model, $P = 0.05$), with the odds of HEV infection being significantly reduced at 71°C compared to that at 62°C and 68°C (5 and 3.7, respectively). Neither the duration of heating nor the interaction between temperature and heating time was significant ($P > 0.05$). Conversely, the time to first detection of anti-HEV antibodies was significantly delayed for the highest temperature (71°C) compared to that for 62°C and 68°C.

DISCUSSION

The presence of HEV in food products consumed raw or cooked medium raises the question of HEV thermal stability. Previous studies had addressed this question, but they did not investigate HEV resistance to heat treatments in complex matrices. These previous studies had investigated HEV resistance in aqueous solution (PBS) or in liver samples (12). Considering that food products made with pork liver, such as liver sausages, have several components, including up to 30% fat, the composition of the food product may affect HEV resistance to thermal treatment. Furthermore, there are few data on viral resistance to heat inactivation in

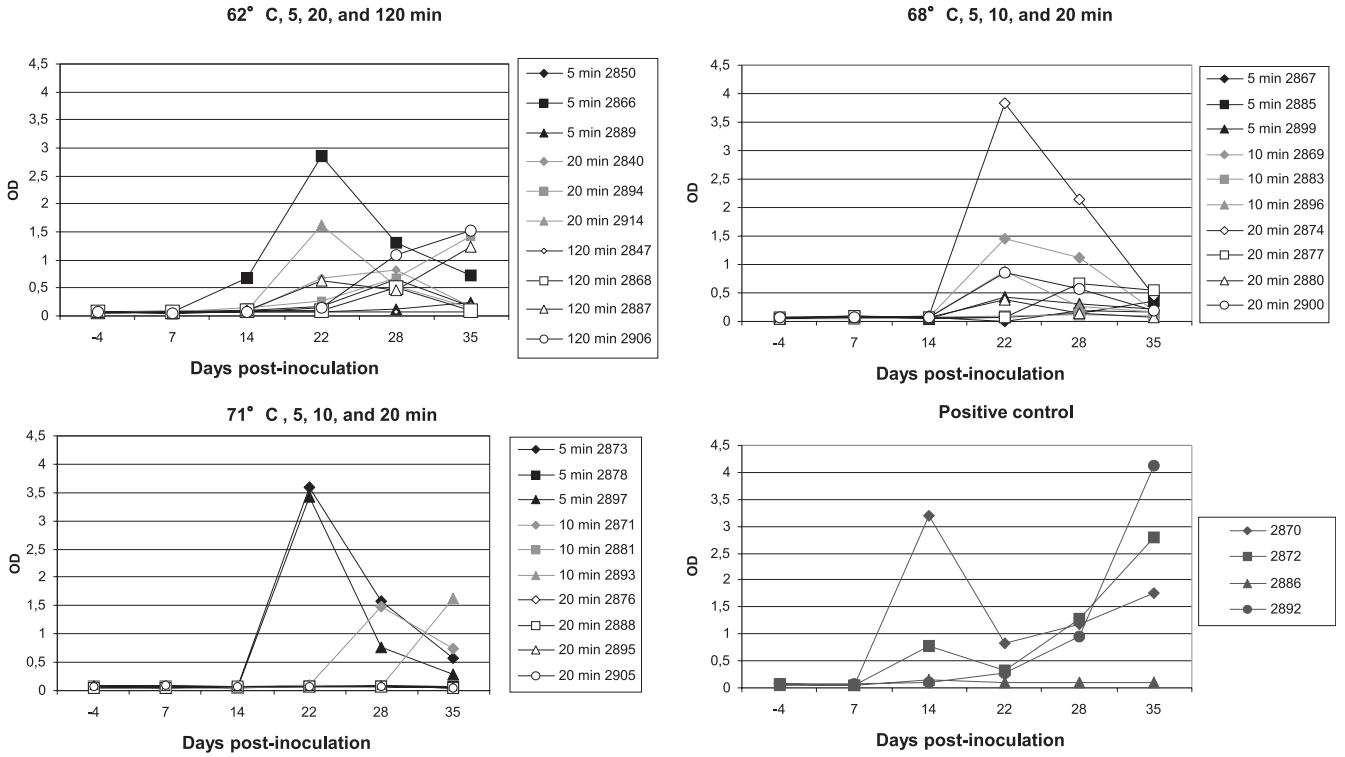

FIG 2 Time course of seroconversion to anti-HEV antibodies in SPF pigs inoculated with the viral suspensions made from the pâté-like preparation after different time/temperature treatments. In each panel, the temperature and duration of incubation of the administered prepared suspensions (one color per duration) are indicated for each pig, along with its identification number (different symbol for each pig in each group).

meat-derived products. Some studies have been performed on hepatitis A virus (HAV) or other enteric viruses such as the norovirus in various types of nonmeat matrices, shellfish (7), dairy products (1), fruits (8), or vegetables (3). However, these results on other viruses cannot be transposed to HEV. Furthermore, in most cases, except in shellfish, viral contaminations occur on the surface of the food product and not deep within the raw material. During HEV infection in swine, the virus replicates inside hepatocytes (liver cells), which results in a high HEV titer in bile; the virus is then shed in feces. Thus, food products containing infected liver are internally contaminated and are not contaminated only on their surface.

In the present study, HEV resistance to thermal treatment was investigated using a food preparation composed of HEV-infected pork liver and other ingredients (fat, spices, etc.) according to industrial recipes. Nine different combinations of time and temperature were applied. All temperatures chosen, 62°C, 68°C, and 71°C, correspond to the thermal treatments applied during industrial food processing. After heat inactivation, a decrease of nearly 3 log units was obtained with the highest temperature of 71°C. In contrast, the lowest RNA titer reduction was observed with the shortest time and lowest temperature combination (5 min/62°C). Thus, a positive effect on the viral genome load reduction is observed with both heating time and temperature. However, it has been shown for several viruses that RT-PCR is unable to discriminate between viruses that are infectious and viruses that have been inactivated (24). In studies with the culturable HAV, quantitative real-time RT-PCR results do not correlate with infectivity (15). Moreover, it has been shown in studies with feline and canine caliciviruses or norovirus that a reduction in infectivity is not always correlated with a decrease in the number of viral genomes detected (9, 10). It is not known whether the presence of HEV RNA corresponds to infectious viruses and therefore if the virus is a threat to human health. As HEV cell culture has been described to have a low efficiency and require a high initial titer, this question can be answered only by using an animal model. Inoculation of pigs with the different preparations showed that only the 20-min/71°C treatment led to the absence of HEV infection of the inoculated animals. Some variability in number of infected animals was observed at each temperature, with more animals infected when the 62°C treatment than the 68°C or 71°C treatment was used, suggesting that a temperature of 62°C had almost no effect on virus inactivation. Due to the small number of pigs used per treatment, it is not possible to clearly estimate the quantity of infectious dose per sample. Variability in the delay before the onset of excretion and the duration of viral excretion was noticeable, and the time to excretion was statistically related to the heating temperature. The observed variability in the duration of excretion may also be related to the possible variability of infectivity of the inocula, even if pigs were inoculated intravenously with the same volume and quantity of virus. For the time/temperature treatment of 10 min/71°C, the observed time to excretion was longer: between 18 and 32 dpi. For practical reasons, the three animals of this group were handled in the same pen as animals in the 10 min/62°C group, which excreted virus earlier, as of 9 dpi. Hence, the possibility of infection of these animals (10 min/71°C) through contact exposure cannot be totally excluded.

The time to and duration of viral excretion could not be formally correlated to the exact quantity of viable HEV received by the pigs, but they can be approximated. These observations were confirmed with the times to seroconversion for each group that were also statistically related to heating temperature. Overall, the results using this pig model of HEV infection showed that there is no clear correlation between the quantity of HEV RNA detected and the presence of infectious virus particles. In comparison to previous studies carried out on HEV thermal resistance in liver homogenates, our results confirm that a temperature of 71°C is necessary to inactivate HEV, but a heating time of 20 min instead of 5 min is required. As observed for other food-transmitted viruses such HAV (6), fat has a protective role and probably contributes to the heat stability of HEV. Thus, food composition and especially fat percentage are important in determining temperature and time of treatment to inactivate HEV.

There are no data on the oral infectious dose of zoonotic HEV currently available. Thus, it is not possible to make any recommendations on the value of HEV GE that would constitute a risk for HEV infection. Furthermore, the present results highlight the fact that there is no clear correlation between HEV genome titer and infectious particles. In the model used here, intravenous inoculation was chosen to ensure the detection of all infectious particles. Oral infection in pigs has been shown to be less efficient (17); thus, the time/temperature scale identified for HEV inactivation in this study can be considered conservative.

In conclusion, the study presented here confirms that heating efficiently inactivates HEV in food products derived from infected pork liver. Such products should be consumed after they have been cooked for at least 20 min to an internal temperature of 71°C.

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